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SUSCEPTIBILITY

Adjunctive Rifampicin Increases Antibiotic Efficacy in Group A Streptococcal Tissue Infection Models

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ABSTRACT Biofilm has recently been highlighted as a complicating feature of necrotizing soft tissue infections (NSTI) caused by *Streptococcus pyogenes* (i.e., group A *Streptococcus* [GAS]) contributing to a persistence of bacteria in tissue despite prolonged antibiotic therapy. Here, we assessed the standard treatment of benzylpenicillin and clindamycin with or without rifampin in a tissue-like setting. Antibiotic efficacy was evaluated by CFU determination in a human organotypic skin model infected for 24 or 48 h with GAS strains isolated from NSTI patients. Antibiotic effect was also evaluated by microcalorimetric metabolic assessment in *in vitro* infections of cellular monolayers providing continuous measurements over time. Adjunctive rifampin resulted in enhanced antibiotic efficacy of bacterial clearance in an organotypic skin tissue model, 97.5% versus 93.9% ($P = 0.006$). Through microcalorimetric measurements, adjunctive rifampin resulted in decreased metabolic activity and extended lag phase for all clinical GAS strains tested ($P < 0.05$). In addition, a case report is presented of adjunctive rifampin treatment in an NSTI case with persistent GAS tissue infection. The findings of this study demonstrate that adjunctive rifampin enhances clearance of GAS biofilm in an *in vitro* tissue infection model.

KEYWORDS rifampicin, *Streptococcus pyogenes*, necrotizing soft tissue infections, biofilm

There is a pressing need to improve outcome in necrotizing soft tissue infections (NSTI) (1). Despite modern treatment, the mortality rate is 18%, and 22% of survivors suffer amputations (2).

Prompt surgical intervention with aggressive tissue debridement combined with appropriate antimicrobial therapy is critical in the management of NSTI (1). The microbiologic etiology of the infections is diverse, but a frequent cause is beta-hemolytic *Streptococcus*, in particular *S. pyogenes*, i.e., group A *Streptococcus* (GAS) (2, 3). Although GAS is uniformly susceptible to penicillin and other β-lactam antibiotics, a combination of β-lactam antibiotics and clindamycin is recommended in patients with severe invasive manifestations to inhibit toxin production and to overcome the Eagle effect (4–6).

Intracellular persistence and biofilm formation have been suggested as potential mechanisms underlying a bacterial persistence and antibiotic treatment failure in GAS pharyngitis and NSTI (7–10). In the study by Siemens et al. (11), biofilm was explored both in NSTI patient biopsy specimens as well as in human organotypic tissue infected with NSTI clinical GAS strains. Biofilm was identified in >30% of patient biopsy specimens, and all clinical isolates readily formed biofilm *in vitro*. Biofilm formation is a known clinical problem associated with chronic wounds and device-associated infections (12). The biofilm

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TABLE 1 Group A streptococcal strains included in the study, *emm* type, and antibiotic susceptibility[a]

Strain (*emm* type)	MIC test	MIC (mg/liter), MBEC (mg/liter)		
		Benzylpenicillin	Clindamycin	Rifampin
2006 (*emm*1)	Broth microdilution	0.008, 4	<0.016, 8	0.125, 4
2006 (*emm*1)[b]	Agar diffusion	0.008, 4	0.12, 8	0.064, 4
2028 (*emm3*)[b]		0.008, 1	0.25, 4	0.032, ≥4
5004 (*emm*28)[b]		0.016, 4	0.12, 4	0.064, ≥4
6004 (*emm*3)[b]		0.008, NA	0.12, NA	0.032, NA
6013 (*emm*1)[b]		0.008, 4	0.12, 8	0.064, ≥4

[a]MBEC shows median values of 3 separate measurements for 2006, while only 1 determination was available for the other indicated strains. Initial MIC testing of 2006 was performed using broth microdilution assay, and these values were used for determination of antibiotic concentrations to be tested. The agar gradient diffusion test was used for MIC determination for the extended strain collection. NA, not available.
[b]Inducible clindamycin resistance (ICR) not detected.

represents a mechanical barrier, and bacteria inside are in a reversible metabolic state with decreased susceptibility toward antibiotics, up to a thousand-fold higher than that needed for planktonic bacteria (13). Several antimicrobial strategies have been proposed, including biofilm dispersal agents as well as use of antibiotics with higher biofilm efficacy. For almost 3 decades, rifampin has been used against biofilm-associated infections of, e.g., orthopedic implants (14). Dental streptococci account for around 10% of prosthetic joint infections (15), which are often treated with penicillin and adjunctive rifampin (16). Rifampin is otherwise mainly used for treatment of *Mycobacterium tuberculosis* infections by virtue of its intracellular efficacy. Gonzalez Moreno et al. tested adjunctive rifampin treatment and found that it has synergistic activity to planktonic and biofilm-embedded GAS on polystyrene surfaces (16). Antibiofilm regimens are difficult to evaluate due to lack of biologically relevant and standardized methods, and antibiofilm properties often depend on the method in use (17).

Here, we sought to explore the *in vitro* effect of adjunctive antibiotic treatment on GAS biofilm formed in the tissue milieu. For this purpose, human skin tissue models were used to assess the efficacy of clindamycin and penicillin with or without rifampin. Rifampin was chosen due to its antibiofilm and intracellular activity, as well as anecdotal clinical efficacy, such as in the case reported below.

RESULTS

Case report. A 52-year-old male underwent extensive debridement and skin grafting of his right arm following *emm*1 GAS NSTI (isolate sensitive to penicillin, clindamycin, rifampin, and doxycycline). The infection was treated with intravenous (i.v.) amoxicillin-clavulanate for 10 days. Five months later, an unhealed area of skin graft on the right elbow yielded *emm*1 GAS again. He was treated with 5 days of oral amoxicillin-clavulanic acid (625 mg three times daily [tds]). Seven months after the initial infection, he was diagnosed with invasive oral squamous cell carcinoma and planned for surgical intervention. At preoperative assessment, swabs of throat and scabbed skin graft still yielded GAS (*emm*1; same sensitivity pattern as previously). With extensive surgery scheduled urgently, daily chlorhexidine body washes and Dermol emollient containing benzalkonium chloride and chlorhexidine dihydrochloride were commenced, plus 10 days of oral amoxicillin-clavulanic acid (625 mg tds). Two days prior to surgery, 7 days into decolonization, skin swabs and throat swabs remained positive for *emm*1 GAS. He was admitted for intensive preemptive therapy with intravenous benzylpenicillin (2.4 g four times daily [qds]) and clindamycin (1.2 g qds). Since the operation could not be delayed further, oral rifampin (600 mg twice daily [bd]) was added for optimal penetration of presumed wound and intraoral biofilm and continued postoperatively until throat swabs and tissue cultures proved negative. The patient was discharged home 10 days postoperatively. Repeated swabs over the next 6 months showed no recurrence of GAS.

Antibiotic effect in *in vitro* infections of organotypic skin tissue. Antimicrobial susceptibility testing of the *emm*1 GAS strain 2006 revealed MIC values indicating sensitivity to the tested antibiotics benzylpenicillin, clindamycin, and rifampin (Table 1). Also, no inducible clindamycin resistance was detected. In contrast, minimum biofilm eradication concentration

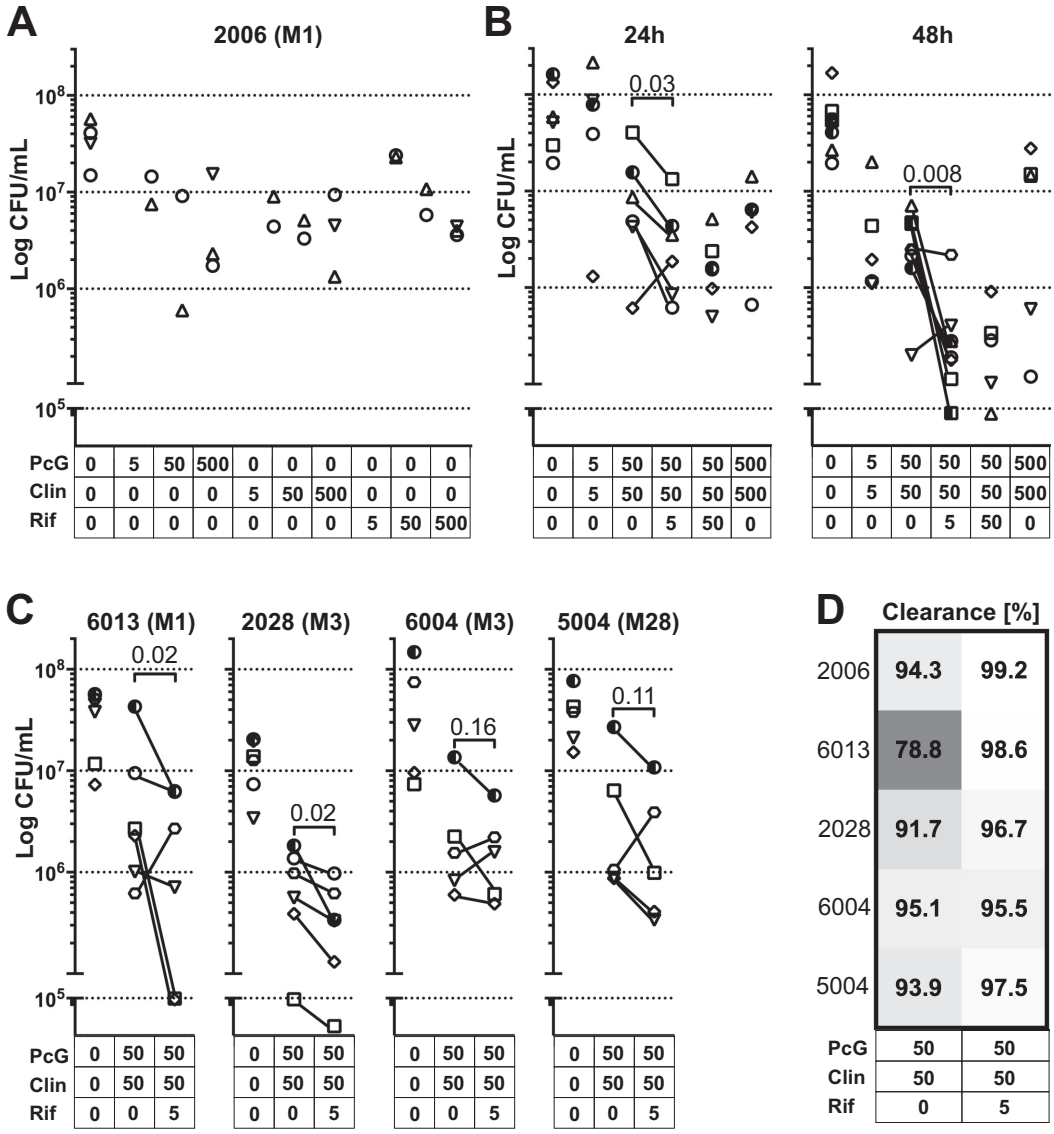

FIG 1 Adjunctive rifampin treatment decreases the bacterial load in organotypic skin tissue infected with group A streptococcal (GAS) strains. Organotypic tissue models were infected with indicated GAS strains of different serotypes (M1, M3, and M28) for 48 h. This was followed by antibiotic monotherapy of benzylpenicillin (PcG), clindamycin (Clin), or rifampin (Rif) for 48 h (A) or combination therapy for 24 h (B) or 48 h (B and C), after which the tissue models were digested and CFU determined. Concentrations of the antibiotics are indicated and represent fold MIC values as related to the MIC of strain 2006. Different symbols indicate experiments performed on separate days. (D) Median clearance of GAS strains after 48 h of combination therapy. Statistical significance between PcG+Clin versus PcG+Clin+Rif combinations was determined by one-tailed Wilcoxon test.

(MBEC) values ranged between 4 and 8 mg/liter, corresponding to 500-, 500-, and 32-fold the MIC of benzylpenicillin, clindamycin, and rifampin, respectively (Table 1). To determine antibiotic efficacy in a tissue-like setting, we utilized human organotypic skin tissue models previously demonstrated to represent a good proxy of GAS biofilm in NSTI patient tissue (11). Benzylpenicillin, clindamycin, and rifampin were tested in dose-response experiments both in mono- and combination therapy using concentrations of 5-, 50-, and 500-fold the MIC value of each respective drug. Monotherapy with either antibiotic reduced the bacterial load in the tissue, but only rifampin displayed a clear dose-dependent reduction (Fig. 1A). However, even concentrations of 500-fold MIC resulted in less than 1-log reduction of CFU (<90% clearance). The combination therapy of benzylpenicillin plus clindamycin (standard treatment) had no effect at 24 h but resulted in 1-log reduction of CFU at 48 h (Fig. 1B). No dose response was noted between 5, 50-, and 500-fold MIC (Fig. 1B). Standard treatment

with adjunctive rifampin achieved a significant reduction of CFU already at 24 h of treatment ($P = 0.03$), which was further reduced after 48 h of treatment ($P = 0.008$) (Fig. 1B). After 48 h of treatment, adjunctive rifampin treatment resulted in a 2-log reduction of CFU and a bacterial clearance rate of 99.2% compared to 94.3% following standard treatment (Fig. 1B).

Next, adjunctive rifampin was tested against four additional GAS strains, all isolated from patients with NSTI and representing common *emm* serotypes (*emm*1, *emm*3, and *emm*28) associated with GAS NSTI (3). The GAS strains were all sensitive to benzylpenicillin, clindamycin, and rifampin (Table 1). After 48 h of infection of the skin tissue model, various CFU were recovered, ranging from 5×10^6 to 2×10^8 without antibiotic treatment (Fig. 1C). Similar to strain 2006, standard treatment resulted in a reduction of the bacterial counts between 78.8 to 95.1% (Fig. 1C and D). Adjunctive rifampin significantly increased clearance compared to standard treatment in three of the five tested strains, reaching 95.5 to 99.2% clearance (Fig. 1D).

Calorimetric assessment of antibiotic effect in *in vitro* infections of cellular monolayers. To reduce experimental variation and to achieve quantitative assessments of bacterial growth in real time, we used continuous calorimetric metabolic measurements. For this purpose, GAS biofilm was established on monolayers of human dermal fibroblasts, after which the biofilm was exposed to different antibiotic treatments for 24 h, followed by continuous calorimetric monitoring for 120 h in antibiotic-free conditions. The standard treatment of benzylpenicillin and clindamycin had relatively poor effect against all 5 strains, as shown by the relatively unaltered metabolic rate (i.e., maximum heat flow) as well as lag extension time (i.e., time to peak) (Fig. 2). In contrast, adjunctive rifampin resulted in significantly lower metabolic rate and an extended lag extension time.

DISCUSSION

In this study, rifampin added to conventional benzylpenicillin and clindamycin therapy showed enhanced efficacy of bacterial clearance in human skin tissue models of GAS biofilm-associated infection. Adjunctive rifampin significantly increased bacterial clearance compared to standard treatment, which only caused a 1-log CFU reduction, even at 500 times the MIC.

A combination therapy of a beta-lactam and clindamycin has long been recommended for treatment of severe invasive GAS infections, such as NSTI and streptococcal toxic shock syndrome (18). A beneficial effect of clindamycin treatment was reported in two population-based active surveillance studies of invasive GAS infections, which both noted reduced mortality in clindamycin-treated patients (19, 20). Similarly, adjunctive clindamycin was found to be associated with improved outcome in patients with invasive GAS infections in a large retrospective multicenter cohort study (21). Also, in a murine model of necrotizing fasciitis, clindamycin was found to significantly reduce the activity of several important virulence factors, including streptolysin O and DNases, and clindamycin treated mice had significantly improved outcome (6). Despite this beneficial outcome, the bacterial burden in the tissue was only marginally reduced. Similarly, studies of patient biopsy specimen material identified viable bacteria present in biofilm communities or intracellularly in tissue biopsy specimens despite prolonged intravenous clindamycin and beta-lactam therapy (6, 7, 11). This is in concordance with the data presented here, where the recommended standard treatment with benzylpenicillin and clindamycin resulted in poor bacterial clearance in a model of GAS biofilm in the tissue milieu.

In contrast, when rifampin was tested as adjunctive therapy, an increased bacterial clearance was achieved. Among the five tested strains, three were more efficiently cleared from the organotypic tissue in the presence of adjunctive rifampin, while two strains only showed a trend toward improved clearance. The noted difference in antibiotic efficacy was not linked to the MIC susceptibility profile of the strain but likely reflects the growth and biofilm properties of the individual strains in the models used. The complex three-dimensional tissue models are inherently associated with fine variations in stratification and cellular composition, which could influence biofilm formation as well as antibiotic distribution in the tissue and thereby contribute to the noted experimental variation.

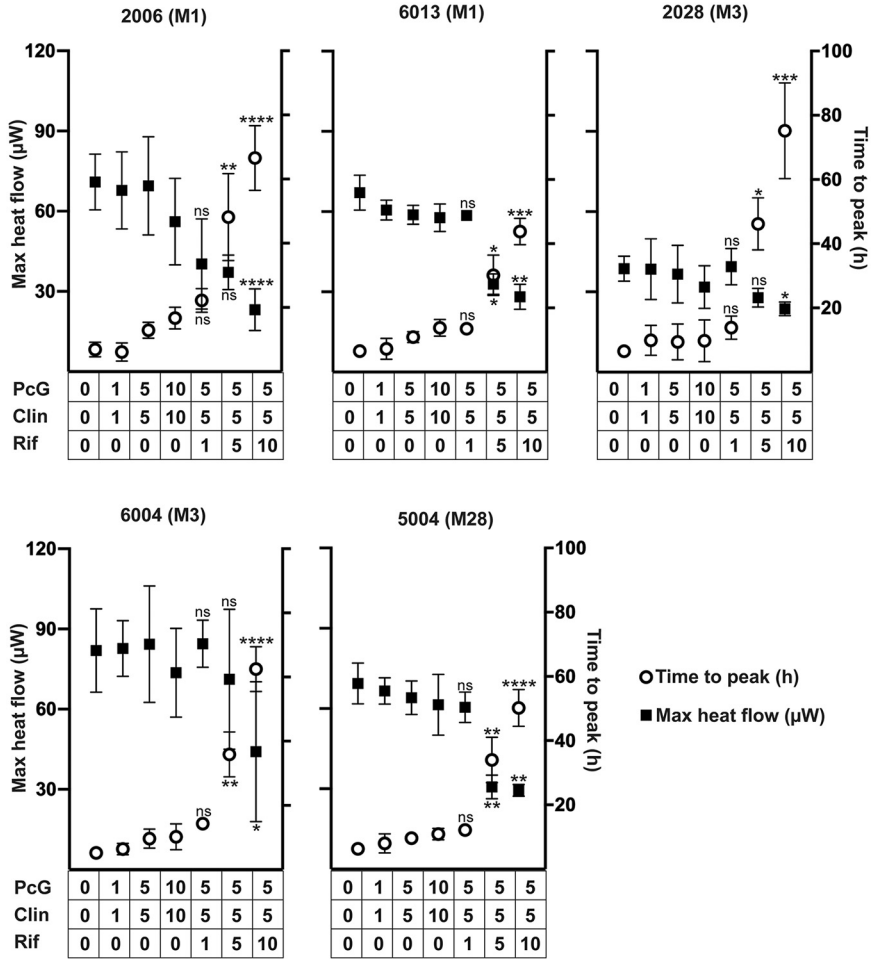

FIG 2 Continuous calorimetric assessment of group A *Streptococcus* (GAS)-infected dermal fibroblasts demonstrates increased treatment effect with adjunctive rifampin. Monolayers of human dermal fibroblasts were infected with indicated GAS strains for 24 h, after which they were exposed to antibiotics for 24 h. Benzylpenicillin G (PcG), clindamycin (Clin), and rifampin (Rif) were used at various concentrations indicated as fold MIC values related to the MIC value of strain 2006. Bacterial growth after antibiotic treatment was assessed by continuous calorimetric monitoring for 120 h in antibiotic-free conditions. The results are shown as maximum metabolic activity (i.e., maximum heat flow [μW]) as well as time to peak metabolic activity (hours). The graphs show mean ± SD of 3 experiments performed in duplicates. Statistical significance was determined by Kruskal-Wallis test and Dunn's test for multiple comparison; comparison of each data set of each data set with adjunctive rifampin to standard treatment (i.e., PcG 5; Clin 5). ns, not significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

To address this and obtain more quantitative data, a calorimetric metabolic screening was conducted on biofilm formed on fibroblast cell monolayers. Such calorimetric measurements were recently reported to efficiently predict antibiotic activity (22). The results obtained were reproducible and highly informative, as quantitative measures of bacterial growth dynamics were obtained for extended time periods. Using this setup, a significant effect of adjunctive rifampin was confirmed for all five strains, albeit with slightly different profiles and susceptibility. The underlying mechanism to these variations remains to be elucidated through studies dissecting the biofilm composition and functionality. It will also be of interest to test whether the bacterial burden can be further reduced by repeat dosages of antibiotics.

This study has a focus on NSTI and particularly on the concept that the tissue milieu triggers GAS biofilm formation. For this purpose, three-dimensional organotypic skin models were used. These models have proven to be robust tools that support streptococcal infection with high resemblance to the local infectious tissue settings in NSTI patients, including biofilm formation (11, 23, 24). Our data here also demonstrate their

usefulness for intervention studies *in vitro* in a human tissue-like setting. However, it should be noted that while the organotypic skin recapitulates key features of real skin with a protective epithelium and dermal layer, it represents a simplistic model of NSTI, as it does not feature the deeper tissue layers of soft tissue and fascia.

One relevant aspect of rifampin treatment is its many side effects. Most problematic is the fact that rifampin is the most potent known inducer of the cytochrome P450 system, which increases the metabolism of many other drugs, complicating its use for multimedicated patients. For NSTI patients typically treated in the intensive care unit, rifampin is likely to be introduced in the postacute phase and particularly in cases complicated by bacterial persistence despite surgical debridement and treatment with beta-lactams and clindamycin. In the case report described here, the GAS infection persisted for up to 7 months despite repeated β-lactam treatment and was eventually resolved after a combination of benzylpenicillin, clindamycin, and rifampin. This case illustrates a complicated course due to long-term bacterial carriage and tissue persistence after an invasive GAS infection. Although there are scarce data available on the prevalence of this complication, anecdotal cases and our data warrant increased attention on antibiotic failure contributing to bacterial persistence.

The importance of adjunctive antimicrobial strategies in streptococcal NSTI is further emphasized by the emerging resistance in GAS strains. Recently, clindamycin resistance was found to be common (31%) in NSTI caused by beta-hemolytic *Streptococcus*, and an association between clindamycin resistance and increased need for extremity amputations was noted (25). Also, mutations in penicillin-binding proteins pbp2x have been reported in GAS and shown to confer reduced susceptibility to β-lactam antibiotics (26, 27).

Conclusion. The findings of this *in vitro* study demonstrate that adjunctive rifampin significantly improved killing of GAS biofilm formed in a human skin tissue model compared to the conventional beta-lactam and clindamycin antibiotic regimen. The clinical efficacy of adjunctive rifampin in GAS NSTI remains to be proven in a clinical trial. However, the results of this study support the consideration of adjunctive rifampin in GAS NSTI cases with signs of bacterial persistence.

MATERIALS AND METHODS

Bacterial strains and culture conditions. GAS strains isolated from patients with NSTI enrolled in the INFECT patient cohort (2) (clinicaltrials.gov ID NCT01790698) were used (Table 1). One strain (2006; *emm*1) was chosen as reference strain, as it has previously been studied extensively regarding biofilm, virulence, and tissue pathology (11, 23, 28). The strains were cultured overnight (16 h) under static conditions and normoxia in Todd-Hewitt medium supplemented with 1.5% yeast extract.

MIC and inducible clindamycin resistance. The initial determination of MIC was performed by broth microdilution assay (29) but with Mueller-Hinton-F broth (MH-F) (30). Later, MIC determination was performed by agar gradient diffusion (MIC test strip [MTS]; Liofilchem, Italy) according to the manufacturer's instructions. Isolates were screened for inducible clindamycin resistance by the double-disk diffusion method (31).

Minimum biofilm eradication concentration. MBEC was determined by a modification of the broth microdilution assay. Biofilm was formed by incubating bacterial suspensions overnight at 35°C, ambient air, in wells of a microtiter plate using tryptic soy broth containing 1% glucose as growth medium. After removal of the growth media and rinsing the biofilms formed on the bottom of the microtiter wells with sterile phosphate-buffered saline (PBS), antibiotics were added in MH-F broth in 2-fold serial dilutions (30). Following overnight incubation and PBS wash, fresh broth without antibiotics was added. The biofilms were disintegrated by sonication, and the resulting suspensions were transferred to a fresh microtiter plate and incubated overnight. The MBEC was determined as the lowest concentration of antimicrobial agent resulting in no growth evident by visual assessment.

***In vitro* infection and antibiotic treatment using an organotypic skin tissue model.** The organotypic three-dimensional skin tissue model is set up using collagen, human keratinocytes, and dermal fibroblasts as detailed in reference 11. The models were infected with 1×10^6 CFU and incubated for 48 h at 37°C, 5% CO_2 to allow biofilm to be formed, after which the medium was replaced with fresh medium containing antibiotics for either 24 or 48 h. Benzylpenicillin (Sigma-Aldrich), clindamycin (Sigma-Aldrich), and rifampin (Sigma-Aldrich) were tested at different concentrations and combinations. After completed antibiotic treatment, the tissue was digested using collagenase A (1.5 mg/ml; Roche Diagnostics) for 30 min at 37°C during agitation using magnets to allow for CFU determination on blood agar plates. Biofilm formation was confirmed in infected models through confocal microscopy of sections immunostained for GAS, extracellular DNA, Nile red, and wheat germ agglutinin (WGA) as previously detailed (11).

Preparation of surface-attached biofilms on fibroblast monolayers and antibiotic treatment. Dermal fibroblasts (4×10^4) were grown in plastic inserts at 37°C, 5% CO_2 (activated calWells; Symcel, Sweden) in EpiLife medium (Invitrogen) supplemented with human keratinocyte growth serum (Invitrogen). At 70%

confluence, 5×10^3 CFU/insert of the bacterial strains were inoculated in EpiLife and allowed to establish biofilm for 24 h. Biofilm formation was indicated by bacterial aggregates at light microscopy as well as by confocal microscopy of samples immunostained for WGA and DAPI (4′,6-diamidino-2-phenylindole) to visualize bacterial chains as well extracellular DNA. At 24 h, medium was replaced with medium containing antibiotics and incubated for another 24 h. After this, the medium was replaced with Todd-Hewitt broth, and microcalorimetric measurements commenced.

Microcalorimetric assay. Microcalorimetric measurements were carried out using the CalScreener according to the manufacturer's instructions (Symcel AB, Sweden). CalWells with prepared samples (32×) and thermodynamic references (16×) were transferred to sterile titanium vials and sealed with an individual lid to 40 cNm torque. Samples were preheated and equilibrated in two steps (10 min and 20 min, respectively) before introduction into the measuring chamber; after equilibration for 1 to 2 h, the individual heat flow (μW) per insert was recorded at 42-s intervals for 120 h. Results were analyzed using calView and calData software (Symcel AB, Sweden).

Statistical analysis. Significance was determined by one-tailed Wilcoxon test or Kruskal-Wallis test with Dunn's multiple comparisons (for microcalorimetric measurements) using GraphPad Prism v7 software (GraphPad, San Diego, CA). A P value of <0.05 was considered significant.

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